ATTACHMENT B

ANNUAL PROGRESS REPORT FOR FY[redacted]
Autonomous Pathogen Detector, LLNL

PROJECT TITLE: Counter-BW Point Detectors DOE/HQ PMIS)
DOE/HQ PROJECT NUMBER: CB04LL LAB/CONTRACTOR: LLNL
B&R CODE: GC0404 DATE: [redacted]
PRINCIPAL INVESTIGATOR(S): Dr. R. Mariella Jr., LLNL, 925-422-8905
HQ PROJECT MANAGER: (O'Connell or Stoutland, NN- 20, (202) 586-3263)

Progress

We met our planned milestones and deliverables for FY[redacted] This included designing, building, and field testing of a 10-chamber, real-time, automated PCR system, which we call ANAA (Advanced Nucleic-Acid Analyzer). To the best of our knowledge, this system outperformed all others, during the DoD Joint Field Trials IV, held at Dugway Proving Grounds, [redacted] for the detection, identification, and quantification of unknown organisms (Each unknown consisted of none, one or two possible simulants from the set of four simulants:

- the spore *B. globigii, (B.g.)* in concentrations ranging from 10^2/ml to 10^5/ml;
- the vegetative bacterium, *Erwinia herbicola,* in concentrations ranging from 10^2/ml to 10^5/ml;
- the RNA virus, MS2, in concentrations ranging from 10^4/ml to 10^7/ml;
- and the protein, ovalbumin. We did not attempt to detect ovalbumin.).

We have built and tested the miniature fluidics and control system (hardware and computer control software) which extracts aliquots of aqueous sample from the Research International aerosol collector and mixes each aliquot with reagents, preparatory to flow analysis. Our experiments have focused on measuring the accuracy and reproducibility of these automated processes.

We have built and tested all the necessary components to demonstrate our first version of an autonomous pathogen detector. A single computer now can control the aerosol sampler, the fluidics, and the simple flow cytometer. We have not, yet, tested the system with real reagents, only with simulated samples and reagents.

We have also begun a collaboration with Luminex, Inc., in the developent of a multiplexed flow-cytometric assay for pathogens.

Detailed Progress Report

Dating back to [redacted], the LLNL BioInstrumentation team, working closely with the personnel in the Center for Microtechnology, had invented prototypes of miniature silicon-based thermal cyclers for PCR, flow chambers and detection systems for flow cytometry, etched-channel electrophoresis systems, columns, injectors, preconcentrators, and detectors for gas chromatography, and a variety of microactuators and micro-electro-mechanical systems (MEMS). This experience and working on these prototypes with the scientists of the Biology and Biotechnology Research Program were the basis for much of our recent advances in the field of BW defense, both under DOE/CBNP funding and under funding from other sources.

Under LLNL's Engineering funding, followed by LLNL LDRD and DOE/OBER funding, we invented the flow-stream-waveguide technique for light collection in flow cytometry and built several instruments using this principle. Our use of the LLNL field-rugged flow cytometer, miniFlo, to run antibody assays produced both excellent quantification as well as identification against the bacterial simulants *B.g.* and *E.h.* Running an Ab-based assay at JFT III, the LLNL miniFlo achieved the highest performance ever seen against *B.g.*, the Anthrax simulant, at the Joint Field Trials - 100% of all unknowns with *B.g.* correctly identified and quantified and no false positives on blanks at 10^3/ml sensitivity.

In FY[redacted], under the DOE CBNP Program, we have built a miniFlo version with the capability to detect a second color, so that we can use the multiplex beads that are manufactured by Luminex, Inc. In our preliminary studies, we have used two capture beads from a four-bead assay to detect *B.g.* and ovalbumin (Ov). See Figure 1. By adding a third color of fluorescent detection, it is easy to distinguish 16 types of beads, so our path to much higher levels of antibody multiplexing is clear.

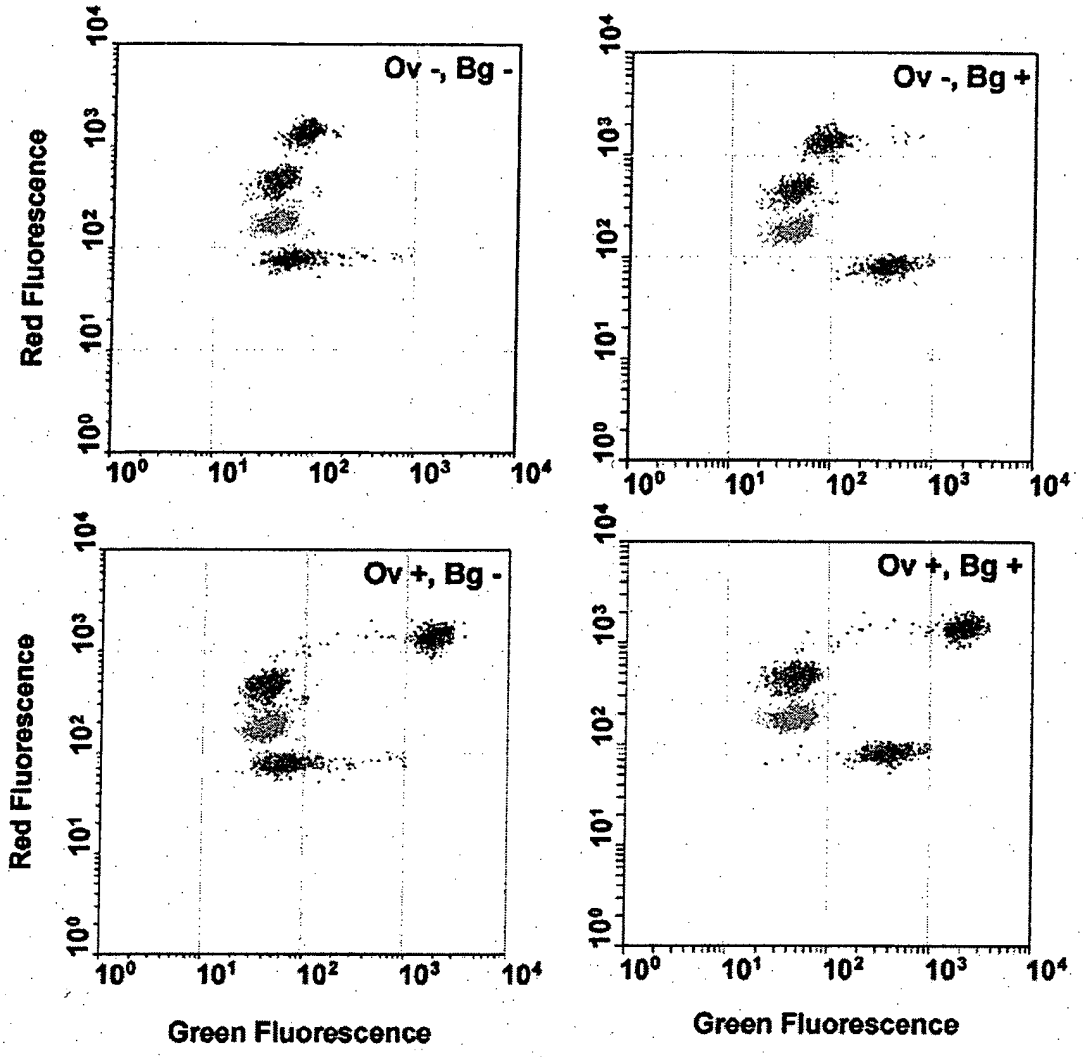

Figure 1. Plots of data from the multiplex assay of Bg and Ov. The upper left panel is a blank assay that shows the location in red fluorescence of the 4 capture beads. They are distinguished from top to bottom by decreasing levels of red fluorescence. In addition the bottom bead is coated with a capture assay for *B.g.* spores and the top bead is coated for Ov. The upper right panel shows a positive for Bg which is indicated by an increase in green fluorescence on that bead alone, while the bottom left panel depicts a positive for Ov. The bottom right is a multiplex determination of *B.g.* and Ov. By adding a yellow fluorescence to the capture beads we have demonstrated the ability to distinguish up to 16 capture bead types.

Under LLNL's Engineering funding and then three years of follow-on funding from DARPA, LLNL has designed, built, and with the collaboration of Roche Pharmaceuticals, Inc., successfully operated the world's first battery-powered, hand-held PCR and briefcase-

sized, real-time PCR instruments, based on thermal cycling chambers made from etched and fusion-bonded silicon. Under CMO/DOE funding, we are collaborating with scientists at the Naval Medical Research Institute (NMRI) to build PCR instrumentation and develop sample preparation protocols. This cooperation between agencies has allowed us to accelerate our original DOE CBNP FY[redacted] milestones, while decreasing the cost from each agency. We have most recently built and field-tested a 10-chamber real-time PCR system, which we have called the Automated Nucleic-Acid Analysis System (ANAA), which includes automated sample preparation using a Cavro, Inc., pipetting robot with a precision physical coupling/registration mechanism to locate the 10 PCR inserts relative to the robot, and with our custom software for automatic calling of positives. Figure 2 shows Jennifer Boyd, of the Dahlgren NSWC, who ran the Navy's Port/Airbase Advanced Concept Technology Demonstration (ACTD) "Portal Shield", with the ANAA system running at DPG in Sept.[redacted]. In the background is the briefcase PCR instrument.

Both the briefcase instrument and the 10-chamber version utilize the Roche-patented Taqman® assay, which adds a second level of confirmation to the PCR assay. PCR normally derives its selectivity from the hybridization required for its primers to anneal to the target sequence before the amplification process can occur. The Taqman assay adds an independent hybridization step with a separate probe that anneals onto the sequence being amplified. During the PCR amplification, the endonuclease activity of the enzyme cuts the Taqman probe into separate nucleotides, releasing a fluorescent label from its nearby quencher, causing an increase in fluorescence as the amplification proceeds. Both the briefcase and the 10-chamber instruments monitor the fluorescence from the Taqman probe during every thermal cycle, providing real-time data from the PCR reaction. This is particularly important when a rapid assay is valuable, since the time it takes to reach a detectable threshold with the Taqman probe assay is inversely related to the starting concentration of the target organism. That is, you quickly know when there is a high concentration of an agent.

The purpose of the Portal Shield ACTD was primarily to evaluate a network of autonomous Ab-based detectors known as Mark II. The operation of the LLNL ANAA at the ACTD was in the role of presumptive verification. That is, if the Mark II network detected the presence of a *B.g.* aerosol, then military personnel would fetch a liquid sample that had been collected by a Mark II and deliver it to us. The sample was presumably positive for *B.g.*, and our system was used to verify this. This ANAA software monitored the progress of the Taqman fluorescence and called positives for the operator. The user interface was designed, along with system's documentation, to allow someone not trained in biology to operate the ANAA. We operatied the ANAA during the last two weeks of the ACTD, the ANAA automatically called positives on every sample that we were given, including samples that were collected in the presence of interferents, such as red smoke. PCR analysis of the same samples, when brought back to the LLNL lab and analyzed using Perkin-Elmer commercial PCR instrumentation, confirmed the presence of *B.g.* in every sample.

The more mature Ab-based assay in the Mark II is commercially available, while our 10-chamber PCR system is not yet for sale, so no comparison is truly fair, but we estimate that the sensitivity of the PCR assay was one to three orders of magnitude higher than the Ab-based assay in the Mark II. We still need to build the control electronics so that each thermal-cycling chamber has independent temperature set points, thus being able to perform different assays in each PCR tube. We need to reduce the size of the system, and we need to redesign the entire system to replace the pipetting robot with a miniature fluidic-sample-preparation

unit. We also need either to increase the number of chambers or to add multiplexity to each tube's assay, or both.

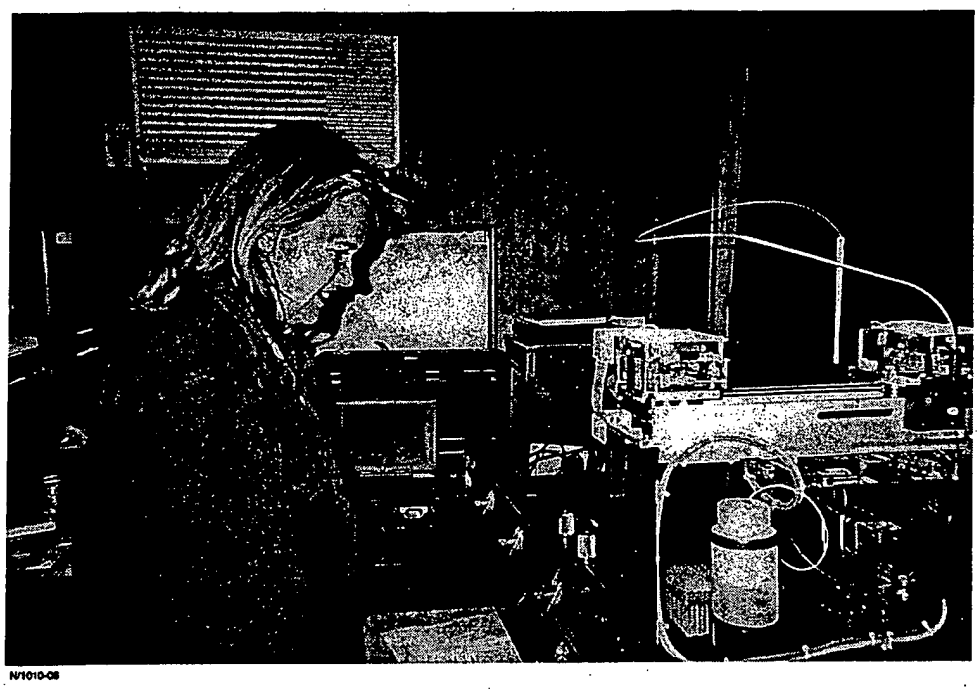

Figure 2. Photograph of Jennifer Boyd, of the Dahlgren NSWC, who ran the "Portal Shield" ACTD, Fall [redacted], shown with the ANAA running at DPG. See text.

Under DOE CBNP funding, we have designed, built, and tested a small breadboarded fluidics system to perform sample preparation for the flow-cytometric Ab-based assay. This fluidics system, connected to miniFlo, is shown in Figure 3. The focus of our current effort is to build a robust system for mixing the sample solution with the Ab solution for incubation, prior to running the assay. This process begins with a solution of pre-mixed Ab reagent. We have not started the experiments that are required to perform the automated reconstitution and subsequent dispensing of lyophilized reagent. We are, however, establishing a collaboration, through the LLNL membership in BSAC, with Prof. Dorian Liepmann, who is working on a similar problem in collaboration with Becton-Dickinson. Over the last 12 months, we have certainly learned that fluidics dealing with environmental samples will clog if there is an orifice dimension under 0.1 mm.

Under the DOE CBNP Program, we have obtained and have performed evaluations of the performance of the leading aerosol collectors, and we either have or are in the process of establishing cooperative development agreements with Research International (RI), SCP Technologies (XM-2®), and MRI (SpinCon®).

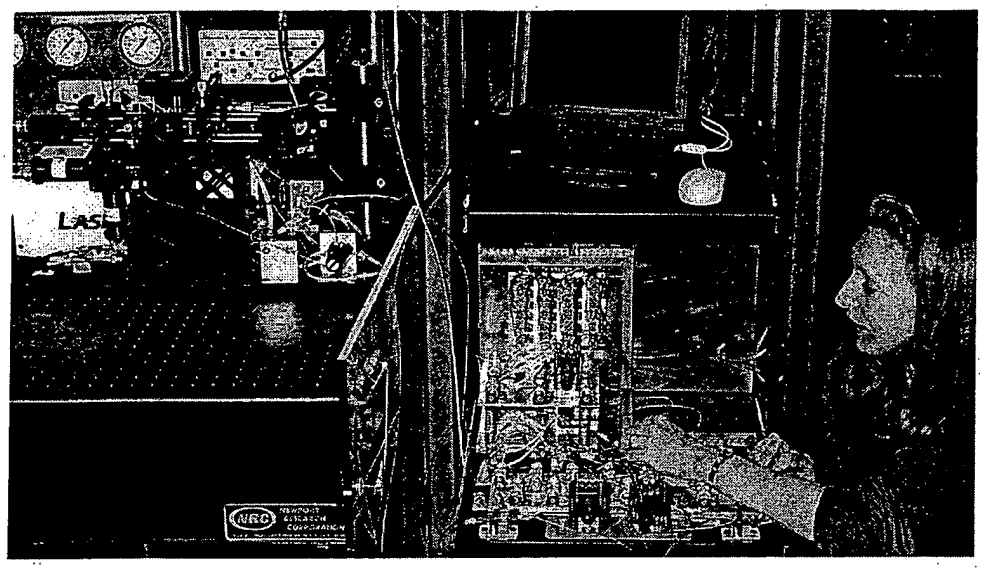

Figure 3. Photograph of Robin Miles, testing her fluidics system connected to miniFlo.

Figure 4 shows our measurements of our measured collection rates for three leading aersosol collectors and the all-glass impinger (AGI). As we mentioned, above, the only unit with a pre-collection fractionation stage is the XM-2. As has been done in other evaluations, we compare the performance of each unit against the AGI, a high-efficiency, low-throughput unit. We are providing these data back to the manufacturers, so that they may improve the performance of their units. As we mentioned, above, under CMO funding we are in the process of demonstrating a new type of PCF, that we expect to provide excellent performance, but with greatly reduced power requirements. Such an improvement would be valuable for a man-portable detection system, according to Lt. Col. Buley, of the JPO-BD.

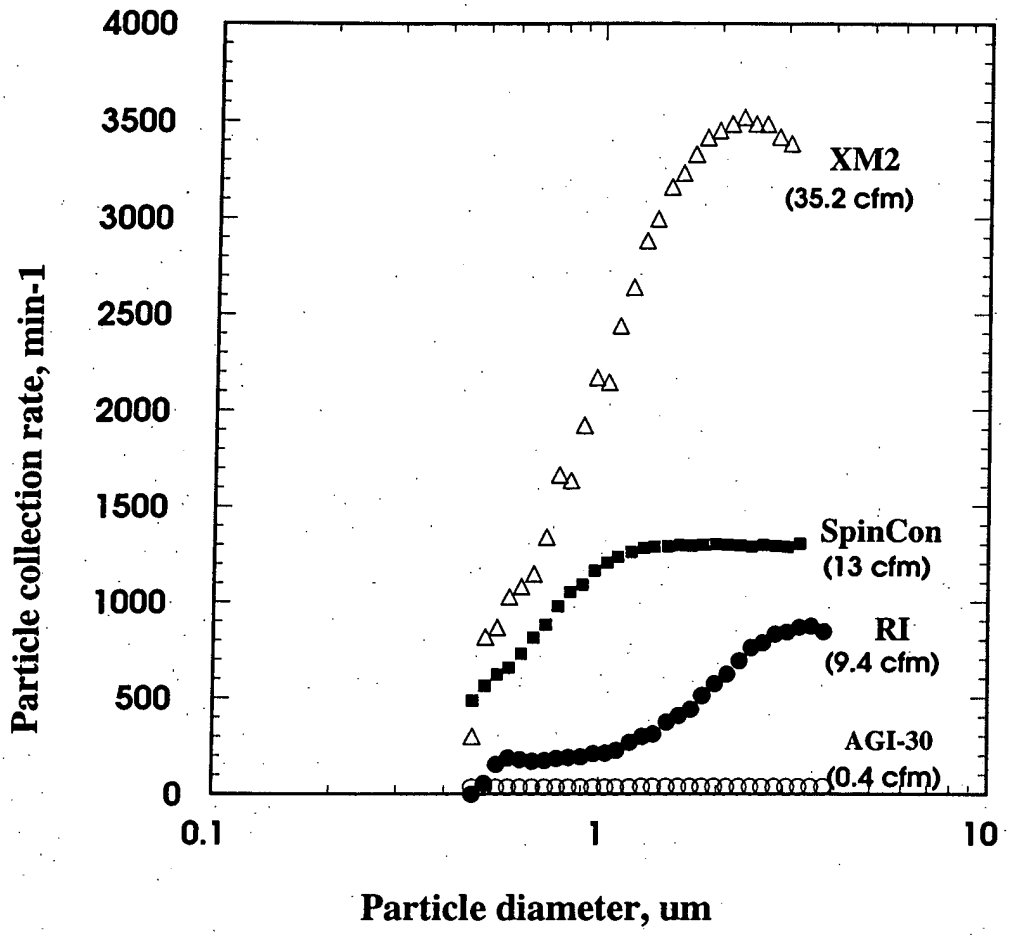

Figure 4, plot of collection performance for three collectors compared with the AGI, using dioctyl sebacate (DOS).

Under funding from the DOE CBNP Program, Dennis Imbro, of the Systems Analysis Thrust Area, has worked closely with us to build a computer model of an autonomous detector/identifier, based on an aerosol collector/concentrator, fluidic transport, and the miniFlo for Ab-based assay. We will continue working with Dr. Imbro, both to refine his model and to help us optimize the performance of our system. This is also of interest to the JPO-BD.

We have met with Greg Frye, from Sandia, and will make sure that our fabrication plans remain compatible with the sample input needs as well as size and power requirements of his instrumentation.

Collaborations

We have earned national recognition for our work, and we either have or are establishing collaborations with other national leaders in CBNP and related technology:

CMO: Maj. Holly Franz, Steve Gotoff
NMRI: Gary Long
ERDEC: Peter Stopa
USAMRIID: Lt. Col. Erik A. Henchal, Assistant Chief, Applied Research Division
Cepheid, Inc.: - Allen Northrup, Greg Kovacs
Research International: Elric Saaski

Regarding mass-spectrometric detection/ID of BW agents, we have met with Drs. Thaiya Krishnamurthy and D. G. Parekh of ERDEC, who are leading this field, and they have expressed interest in connecting their work with ours. Again, due to the [redacted] delivery time for the DOE CBNP Program, it is unlikely that mass spec will be ready in time, but Krishnamurthy participated in JFT IV, this [redacted], and we plan to stay in communication.

TECHNICAL REPORTS/PRESENTATIONS

JFT IV results with the use of the LLNL 10-chamber PCR system, presented to the personnel at Dugway Proving Grounds, presented by Drs. Belgrader and Mariella, [redacted]

R. Mariella Jr. and F. Milanovich participated in the Intelligence Community Field Demo workshop, held at Patrick AFB, [redacted].

R. Mariella Jr. gave a presentation on Microtechnology for Instrumentation at the International Sensors Expo, held in San Jose, [redacted]

On 9 - 10 [redacted], R. Mariella Jr. participated in the NASA review panel for Environmental Monitoring and Control, Washington, D.C., reviewing instrumentation proposals for biodetection on manned space flights.

Presentations to visitors at LLNL:
Gary Resnick (head of R&T of ERDEC)
Jane Alexander (Dep.Dir. of DSO at DARPA)
Admiral Mies from USSTRATCOM
Dr. Jay C. Davis Director Designate Defense Threat Reduction Agency (DTRA)
Lt. Col Niemeyer (USAF Force Protection Battlelab)